Comprehensive Proteomic Analysis of Human Milk-derived Extracellular Vesicles Unveils a Novel Functional Proteome Distinct from Other Milk Components*[S]

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Breast milk contains several macromolecular components with distinctive functions, whereby milk fat globules and casein micelles mainly provide nutrition to the newborn, and whey contains molecules that can stimulate the newborn's developing immune system and gastrointestinal tract. Although extracellular vesicles (EV) have been identified in breast milk, their physiological function and composition has not been addressed in detail. EV are submicron sized vehicles released by cells for intercellular communication via selectively incorporated lipids, nucleic acids, and proteins. Because of the difficulty in separating EV from other milk components, an in-depth analysis of the proteome of human milk-derived EV is lacking. In this study, an extensive LC-MS/MS proteomic analysis was performed of EV that had been purified from breast milk of seven individual donors using a recently established, optimized density-gradient-based EV isolation protocol. A total of 1963 proteins were identified in milk-derived EV, including EV-associated proteins like CD9, Annexin A5, and Flotillin-1, with a remarkable overlap between the different donors. Interestingly, 198 of the identified proteins are not present in the human EV database Vesiclepedia, indicating that milk-derived EV harbor proteins not yet identified in EV of different origin. Similarly, the proteome of milk-derived EV was compared with that of other milk components. For this, data from 38 published milk proteomic studies were combined in order to construct the total milk proteome, which consists of 2698 unique proteins. Remarkably, 633 proteins identified in milk-derived EV have not yet been identified in human milk to date. Interestingly, these novel proteins include proteins involved in regulation of cell growth and controlling inflammatory signaling pathways, suggesting that milk-derived EVs could support the newborn's developing gastrointestinal tract and immune system. Overall, this study provides an expansion of the whole milk proteome and illustrates that milk-derived EV are macromolecular components with a unique functional proteome. ***Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.060426, 3412–3423, 2016.***

Breast milk is a complex body fluid containing a variety of macromolecular components with distinct functions. Breast milk does not only have a nutritional value for the newborn, it also has various bioactive properties, *e.g.* nurturing of commensal bacteria, killing of pathogens, promoting intestinal barrier function development, and supporting development and balancing of the newborn's immune system (1–3). The beneficial effect of breastfeeding is reflected by the observation that breast milk intake results in a reduced risk for developing allergic disorders (2, 4). Although these functional aspects of breast milk have been observed, it is not yet clear which macromolecular components in milk perform these functions and via which molecular pathways they exert these effects.

The composition of milk is generally described as a mixture of macromolecular components which include cells, milk fat globules (MFG)[1], casein micelles, and whey (5–8). Although not mutually exclusive, these major components are often categorized into nutritional components and functional components (9). The primary nutritional components are MFG,

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[1] The abbreviations used are: MFG, Milk fat globules; ANXAs, Annexins; ER, Endoplasmic reticulum; EV, Extracellular vesicles; GO, Gene Ontology; MFGM, Milk fat globule membrane; RABs, Ras-related proteins.

which are the main source of lipids, and caseins, which provide most of the protein. Functionally bioactive properties are mainly allocated to the whey fraction, which contains carbohydrates, antimicrobial proteins, and immunomodulatory factors (6–8).

Apart from these well-described macromolecular structures, breast milk contains extracellular vesicles (EV) as well (10–13). EV are lipid bilayer enclosed vesicles that are released by cells as mediators of intercellular communication (14). EV released by viable cells are very heterogeneous in composition and size (ranging from ~50 nm to >1 μm), with the vast majority <200 nm. EV can originate from either the endosomal route being released from multivesicular bodies as exosomes, or they are released as microvesicles by direct budding from the plasma membrane (15). Although the biogenesis of exosomes and microvesicles is different, once released into the extracellular milieu (as is the case with breast milk) there are no markers to distinguish these subsets of vesicles (16, 17). The molecular composition of EV (functional proteins, nucleic acids and lipids) is regulated by the producing cell and depends on the type and condition of the cell (18). Although for human milk-derived EV some microRNAs and proteins have been identified (12, 19), a detailed characterization of milk-derived EV is lacking. This makes milk-derived EV an understudied macromolecular component of milk.

Proteomic analysis has allowed for the exploration of the protein composition of distinct macromolecular structures. However, in a complex body fluid such as breast milk, which contains a variety of macromolecular components in the presence of a few highly abundant proteins, detection of low abundant proteins is easily obscured. Hence, to unravel the full milk proteome it is essential to define appropriate isolation methods for the individual components followed by high-resolution mass spectrometry. Recently, we developed a protocol to reliably isolate pure EV from other macromolecular structures in milk, allowing in-depth characterization of milk-derived EV (10).

In this study, in-depth proteomic analysis on purified human milk-derived EV from seven individual donors was performed and the first comprehensive proteome for milk-derived EV was established. We identified 198 novel EV-associated proteins not present in the EV database Vesiclepedia. By comparing the milk-derived EV proteome to the total milk proteome, which was manually constructed using previously published proteomics data of various isolated milk components, 633 proteins were identified in the milk-derived EV proteome that have not been identified in milk previously. Based on GO analysis, these 633 newly identified milk proteins have distinct functions from other milk proteins, indicating that milk-derived EV are a separate bioactive component of breast milk.

EXPERIMENTAL PROCEDURES

Breast Milk Collection—Breast milk was collected as previously described (10). Briefly, fresh, mature milk samples were collected by seven healthy mothers (between 3 and 9 months after delivery) who were not actively terminating breast feeding. Additional donor information can be found in Table I. Milk was prevented from cooling down and transported to the lab in order to start EV isolation within 20 min after collection. Informed consent was signed by all donors and this study was approved by the local ethics committee.

Isolation of Milk-derived EV and High-density Complexes—Isolation of milk-derived EV was done as previously described (10). Whole milk was centrifuged at RT for 10 min at 3000 × *g* (Beckman Coulter Allegra X-12R, Fullerton, CA) (Fig. 1*A*) in which a white pellet and cream layer were formed. The cream layer, containing MFG, was removed and milk supernatant was harvested without disturbing the cell pellet. Supernatant was transferred to new tubes and centrifuged at 3000 × *g* at RT. The supernatant was stored at 80 °C until further processing. Later, frozen supernatant was thawed and transferred immediately to polyallomer SW40 tubes or SW28 tubes (Beckman Coulter) and centrifuged at 5000 × *g* for 30 min at 4 °C and subsequently at 10,000 × *g*. Next, 6.5 ml aliquots of the 10,000 × *g* supernatant were loaded on top of individual gradients in a SW40 tube. For donor 1, a total of 6.5 ml was used, and for donors 2–7 the total volume was 32.5 ml (Table I). The gradient consisted of 15 successive layered sucrose fractions of 350 μl (ranging from 2.0 M to 0.4 M sucrose, with a decrement of 0.114 M per fraction) on top of 700 μl 2.5 M sucrose. Ultracentrifugation was done at 192,000 × *g* (in a Beckman Coulter Optima L-90K with a SW40 rotor) for 15–18h. After ultracentrifugation, fractions of 500 μl were harvested from the density gradient starting from the bottom of the SW40 tube and collected in Eppendorf tubes. The density of the collected fraction was measured by refractometry in order to identify the fractions containing milk-derived EV (densities of 1.12–1.18 g/ml sucrose) or high-density complexes (bottom 3 fractions with densities 1.23–1.28 g/ml). After collection of all fractions, the residual pellet was resuspended in 500 μl PBS. Fractions containing EV and fractions containing high-density complexes were pooled (the resuspended pellet was added to the high density complex sample). Pooled fractions were transferred to SW28 tubes and diluted with PBS and samples were pelleted at 100,000 × *g* for 65 min. After centrifugation, supernatant was removed and pellets were stored at −80 °C.

Protein Extraction—For protein isolation, pellets were suspended in lysis buffer containing 8 M urea in 50 mM ammonium bicarbonate, 1 tablet of protease inhibitors Complete mini (Roche ref. 11836170001, Sigma, St. Louis, MO) and 1 tablet of PhosSTOP phosphatase inhibitor mixture (Roche ref. 04906845001). Sample sonication was carried out using a UP100H sonicator (Hielscher Ultrasound Technology, Teltow, Germany) for 6 cycles of 1 s pulse with a 30 s gap in between at 60% amplitude. Clear supernatant was collected after centrifugation of the sample at 20,000 × *g* for 15 min at 4 °C for isolation of soluble proteins.

SDS-PAGE, In-gel Digestion and Western Blotting—For high-resolution LC-MS/MS analysis samples were further processed after sonication. For this, 20 μl or 40 μl of each sample was added to 10 μl 200 mM DTT (Sigma ref. 43815) and 10 μl sample buffer (Bio-Rad ref. 161–0791, Hercules, CA) and incubated for 5 min at 95 °C. After a short centrifugation step, the samples were separated on a 12% Tris-HCl gel next to a protein ladder (Thermo ref. 10747012, Waltham, MA). The gels were fixed with 50% ethanol/10% acetic acid, stained with Gelcode Blue Stain (Thermo ref. 24592) and destained with MilliQ (Millipore, Billerica, MA) (Fig. 1*B*). Each lane was excised in 6 or 7 bands for in gel digestion using trypsin. After extraction with 100% acetonitrile the samples were dried and reconstituted in 40 μl of 10% formic acid/5% DMSO (Sigma St. Louis, MO).

For Western blotting (Fig. 4, Fig. 5*B,* and Fig. 6*B*), 1 ml of the pooled fractions containing EV and pooled fractions containing high-density complexes from donors 4 to 7 were taken and transferred to SW60 tubes (Beckman Coulter) and diluted with PBS. Next, samples were pelleted at 100,000 × *g* for 65 min (in a Beckman Coulter Optima L-90K with a SW60 rotor) and pellets were taken up in reducing buffer (containing 350 mM β-mercaptoethanol) for EHD3 detection, or nonreducing sample buffer for the detection of flottilin-1, CD9, OLAH, PTHLH and MPZL1. Samples were loaded on a 4–20% TGX-Criterion gel (Bio-Rad, Hercules, CA) and separated proteins were transferred to PVDF membranes followed by blocking in PBS containing 0.2% fish skin gelatin and 0.1% Tween-20. Proteins of interest were detected by immunoblotting using mouse anti-human flotillin-1 (clone 18, BD Biosciences, San Jose, CA), mouse anti-human CD9 (HI9a, BioLegend, Fell, Germany), rabbit anti-human OLAH (HPA037948, Atlas Antibodies, Stockholm, Sweden), rabbit anti-human PTHLH (PA5–40799, Thermo Fisher, Rockford, IL), mouse anti-human MPZL1 (SAB1406763, Sigma), mouse anti-human EHD3 (ab194512, Abcam, Cambridge, UK). Secondary antibodies used were goat anti-mouse-HRP (Jackson Immuno Research, Suffolk, UK) and goat anti-rabbit-HRP (Dako, Heverlee, Belgium). Labeled antibodies were detected using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Landsmeer, Netherlands) and blots were imaged using a ChemiDoc XRS (Bio-Rad).

High-resolution LC-MS/MS Analysis—Samples were individually acquired in two separate experiments (milk-derived EV or non-floating complexes from donor 1 and 2 in the first experiment. Milk-derived EV or non-floating complexes from donor 3 to 7 in the second experiment). Experiment 1 was analyzed by nanoflow LC-MS/MS using a Proxeon EASY-nLC 1000 (Thermo Scientific, Odense, Denmark) with an analytical column heater (40 °C) coupled to an LTQ-Orbitrap Elite. In gel digestion fractions were dried, reconstituted in 10% FA and delivered to a trap column (ReproSil C18, (Dr Maisch GmbH, Ammerbuch, Germany); 20 mm × 100 μm inner diameter, packed in-house (at the Netherlands Proteomics Centre) at 5 μl/min in 100% solvent A (0.1 M acetic acid in water). Next, peptides eluted from the trap column were loaded onto an analytical column (Poroshell 120 EC-C18 (Zorbax, Agilent Technologies); 40 cm length, 50 μm inner diameter, packed in-house) at ~100 nl/min in a 60 min gradient from 0 to 40% solvent B (0.1 M acetic acid in 8:2 (v/v) ACN/water). The eluent was sprayed via distal coated emitter tips butt-connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS. Full-scan MS spectra (from *m/z* 350 to 1500) were acquired in the Orbitrap with a resolution of 60,000 Full width at half maximum (FHMW) at 400 *m/z* using an AGC setting of 1e6 ions. After the survey scans, the 10 most intense precursors were selected for subsequent decision tree-based ion trap CID or ETD fragmentation. Experiment 2 was analyzed using a Q-Exactive instrument (Thermo Scientific, Bremen) connected to an Agilent 1290 Infinity LC system with an analytical column heater (40 °C), a trap column of 20 mm × 100 μm ID Reprosil C18 (Dr Maisch) and a 400 mm × 50 μm ID Poroshell C18 analytical column (Zorbax, Agilent Technologies), all packed in-house. Solvent A consisted of 0.1 M acetic acid (Merck) in Milli-Q (Millipore), whereas solvent B consisted of 0.1 M acetic acid in 80% acetonitrile (Biosolve). Trapping was performed at a flow rate of 5 μl/min for 10 min and peptides were eluted using a passively split flow of 100 nl/min for 60 min. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS. For the MS analysis the ten most intense ions in the survey scan (350 to 1500 *m/z*, resolution 35,000, AGC target 3e6) were subjected to HCD fragmentation (resolution 17,500, AGC target 5e4), with the normalized collision energy set to 25% for HCD. The signal threshold for triggering an MS/MS event was set to 500 counts. The low mass cut-off for HCD was set to 180 *m/z*. Charge state screening was enabled, and precursors with unknown charge state or a charge state of 1 were excluded. Dynamic exclusion was enabled (exclusion size list 500, exclusion duration 15 s).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (20) partner repository with the data set identifier PXD004423.

Data Processing—MS raw data were processed with Proteome Discoverer (version 1.3, Thermo Scientific). Generated peak lists were searched against the Uniprot database; Homo sapiens (July 2014, 546,121 entries), supplemented with frequently observed contaminants using Mascot software version 2.3 (Matrix Science, UK). Trypsin was chosen as enzyme and two missed cleavages were allowed. Carbamidomethylation (C) was set as a fixed modification and oxidation (M) was set as variable modification. The searches were performed using a peptide mass tolerance of 50 ppm and a product ion tolerance of 0.05 Da (HCD), followed by data filtering using percolator, resulting in 1% false discovery rate (FDR). Only ranked 1 PSMs with Mascot scores >20 were accepted. Keratins were not considered contamination and therefore were not removed from the data set, because mammary epithelial cells (as possible EV producers) express keratins. The data from this study can be found in supplemental File S1 and has been submitted to the Vesiclepedia database, http://www.microvesicles.org/(accession number: Vesiclepedia_574).

Experimental Design and Statistical Rationale—Mass spectrometry was performed on seven biological replicates with milk-derived EV and high-density complexes, whereas Western blot analysis was performed on four biological replicates. Functional enrichment analysis for Gene Ontology (GO) terms and comparison of data sets (Fig. 2, Fig. 3, Fig. 5, and Fig. 6) was done using FunRich (with the human FunRich database as background; as recent as November 2015) (21). Comparison between this study and EV database Vesiclepedia was done by importing the online protein data from the Vesiclepedia database (as recent as November 2015) into FunRich. This data set included all proteins listed in Vesiclepedia (from all tissues and cell types; all cell lines; all isolation methods; all detection methods; all vesicle types). GO analysis of annotated proteins was performed for cellular component, molecular function, biological processes and site of expression. Enriched terms were ranked by *p* value (Hypergeometric test) by FunRich. GraphPad Prism software (La Jolla, CA) was used to plot the graphs when comparing data sets. In order to show the highest diversity of enriched GO terms, duplicate or highly similar terms were removed, selecting those with the highest statistical power or with the highest number of genes in the background data set. For cellular component in Fig. 2*B* and Fig. 6*C*, GO terms for 11 major cellular components were selected in order to limit results (nucleus, ribosome, vesicle (named exosomes in FunRich), endoplasmic reticulum (ER), Golgi apparatus, cytoskeleton, mitochondrion, cytoplasm, lysosome, centrosome, and plasma membrane) and supplemented with extracellular to include secreted proteins. For site of expression analysis, primary cells reported to reside in the mammary gland or cells known to be present in the circulation were selected. Cell lines, other tissues than breast tissue, or fluids were excluded from the analysis. In comparisons for molecular function and biological process between milk proteins or milk-derived EV proteins in Fig. 6*C*, enriched GO terms that were significant (GO terms with a *p* value <0.05 corrected for multiple comparisons by hyper geometric test) in at least one data set were selected and compared with the other data set. The number of genes in the background data set was at least 15 in order to remove underrepresented GO terms.

For statistical comparison of data sets within a single GO-term (Fig. 2*B* and Fig. 6*C*), the number of proteins linking to the GO term were tested for significant difference in distribution with chi-square test

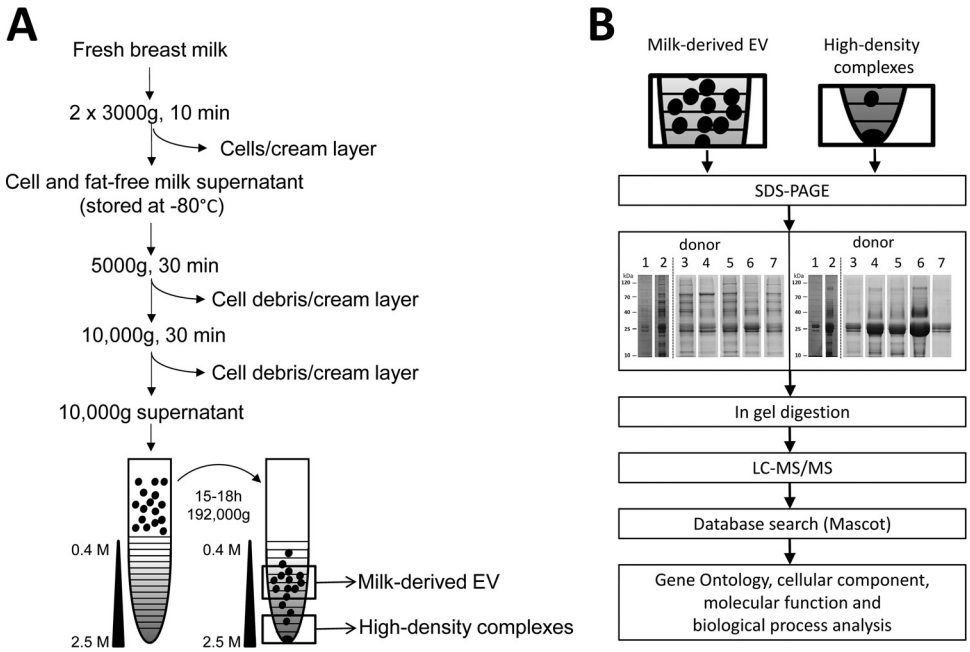

FIG. 1. **Workflow for the isolation and proteomic analysis of milk-derived EV.** *A*, Workflow for the isolation of milk-derived EV and high-density complexes. Breast milk from seven donors was processed individually and subjected to differential centrifugation in order to remove live cells, cell debris and cream (containing MFG). Top down ultracentrifugation was performed whereby 10,000 g milk supernatant was loaded onto a sucrose gradient followed by overnight centrifugation. Individual fractions were collected and fractions 6–9 (with a density of 1.12–1.18 g/ml) were pooled to obtain milk-derived EV, whereas the three bottom fractions plus the pellet were pooled and contained high-density complexes. *B*, Workflow for the processing of milk-derived EV or high-density complexes for in-depth proteomic analysis. Individual samples were subjected to SDS page followed by excision of gel bands for trypsin digestion. Dashed lines indicate that donors 1 and 2 were analyzed in a separate mass spectrometry experiment from donors 3 to 7. Peptides were introduced to the mass-spectrometer and identified proteins were subjected to GO analysis.

using GraphPad prism software. Data sets with a p value <0.05 were regarded as significant.

Comparison of identified proteins between all seven individual donors (Table III) was done using the Venn diagrams web tool from http://bioinformatics.psb.ugent.be/webtools/Venn/.

Construction of the Human Milk Proteome—Previously published raw data was obtained from eight studies, with six primary studies and two review studies that contained a total of 38 individual studies. Supplemental File S2 contains the raw data from these eight studies and supplemental Table S1 contains an overview of the individual 38 studies. Data sets from Beck *et al.* (22) and D'Alessandro *et al.* (23), which summarized previous published work, were used instead of each individual study reviewed. Data sets were converted to the common gene name format in order to construct one data set that could be compared with our data set. Genes listed under multiple (alternative) names were condensed to the common gene name as listed in the UniProt database. Proteins without a common gene name ("N/A"), or entries no longer existing in the UniProt database, or listed under cDNA entries were excluded from the milk proteome data set. The common gene names from all studies were imported into FunRich as a data set and FunRich was used to compare this data set in a Venn diagram.

RESULTS

Isolation of Milk-derived EV and Workflow of the Proteomics Approach—Isolation of milk-derived EV from breast milk of seven donors was performed using the protocol by Zonneveld *et al.* (10) (Fig. 1*A*). First, fat (containing MFG), cells and cellular debris were removed by differential centrifugation resulting in 10,000 g milk supernatant. The milk supernatant was loaded on top of a sucrose gradient and ultracentrifuged to allow for the separation and concentration of EV. This top-down density gradient ultracentrifugation was used because pelleting EV from milk supernatant via 100,000 $\times$ g centrifugation results in an insoluble pellet (10). After ultracentrifugation, individual fractions were collected. To prepare the milk-derived EV sample, EV enriched fractions with densities 1.12 to 1.18 g/ml were pooled. Additionally, high-density fractions (density 1.23 to 1.28 g/ml), enriched in nonmembrane-associated protein complexes, were pooled and used as a control.

Next, the isolated milk-derived EV and high-density complexes from the individual donors were further processed (Fig. 1*B*). Although differences in total protein amount between donors were observed, SDS-PAGE analysis showed large similarities between different donors in the protein patterns obtained for both milk-derived EV and high-density complexes. Milk-derived EV had a more complex banding pattern than high-density complexes, which exhibited a few high abundant proteins (Fig. 1*B*). For each individual donor, protein bands were excised for in gel digestion and these samples were subjected to LC-MS/MS analysis.

Identification of the Milk-derived EV Proteome—In-depth proteomic analysis was performed to determine the proteome

TABLE I

Information regarding age, stage of lactation (months after delivery) and number of pregnancies to a viable gestational age (parity) of the individual donors. Additionally, volumes of milk collected and amount of 10,000 g milk supernatant that was used for further isolation of milk-derived EV and non-floating complexes is given, as described in the experimental procedures

Donor	Age donor	Months after delivery	Parity	Amount of milk (ml)	Amount of 10,000 g sup (ml)
1	35	6	3	10	6,5
2	35	4	1	35	32,5
3	34	4	1	30	32,5
4	32	5	1	33	32,5
5	32	5	1	30	32,5
6	39	9	3	30	32,5
7	34	3	2	30	32,5

of milk-derived EV and high-density complexes. The total number of proteins identified was higher for milk-derived EV than high-density complexes. For milk-derived EV, proteins numbered between 650 and 1615, whereas for high-density complexes this ranged from 194 to 495 individual proteins (Table II). Remarkably, in the milk-derived EV from donor 2 there were more proteins identified, although this donor was similar to the other donors (Table I and high-density complexes in Table II). In total, 1963 unique proteins were identified in milk-derived EV in all samples, whereas 739 unique proteins were determined in the high-density complexes (Fig. 2*A*). To get more insight into the subcellular origin of the identified proteins, functional enrichment analysis for the cellular component was done. These analyses showed that in both milk-derived EV and high-density complexes a high percentage of proteins linked to GO terms like "exosomes" (which are a subclass of extracellular vesicles), "lysosome," and "cytoplasm" (Fig. 2*B*). We next determined the influence of the overlapping proteins for the enrichment analysis (Fig 2*A*: 606 proteins). Within the samples, we compared nonoverlapping proteins (1357 in milk-derived EV and 133 for high-density complexes) to the proteins identified in the entire sample (1963 for milk-derived EV and 739 for high-density complexes). When the overlapping proteins were excluded for the GO analysis, then especially exosomes were significantly lower for milk-derived EV (Fig. 2*B*, left figure). For high-density complexes especially GO-terms "exosomes," "lysosomes," "cytoplasm" and "plasma membrane" were significantly less enriched when the overlapping proteins were excluded for analysis. This suggests that the overlapping proteins most likely are derived from some high-density EV present in the high-density complexes fractions, whereas the 133 proteins unique in the high-density complexes are most likely not EV-associated proteins. Therefore, we selected all proteins identified in the milk-derived EV sample (1963) for further analysis.

Identification of the Origin of Milk-derived EV—In the context of intercellular communication via milk-derived EV between mother and child, it is interesting to consider which maternal cells were the potential producers of milk-derived EV. Apart from cells making up the breast tissue, like mammary epithelial cells and adipocytes, a variety of immune cells have been isolated from breast tissue and milk (5, 24) and all these cells could be the source of milk-derived EV. Via GO analysis we determined the site of expression of the identified EV-proteins (Fig. 3). Interestingly, although GO terms for "breast," "mammary gland," and "mammary epithelium" were significantly enriched in the milk-derived EV proteome, the GO terms related to immune cells like "dendritic cell," "CD4 T cell," "platelet," "monocyte," and "B cell" were most prominently enriched. These findings indicate that milk-derived EV do not only originate from breast tissue but that immune cells likely contribute to the production of these macromolecular components in breast milk.

TABLE II

The number of identified proteins in the milk-derived EV samples and the high-density complexes samples per individual donor

	Number of identified proteins milk-derived EV	Number of identified proteins high-density complexes
Donor 1	857	495
Donor 2	1615	358
Donor 3	817	194
Donor 4	650	157
Donor 5	783	260
Donor 6	814	236
Donor 7	964	304

Identification of Common Milk-derived EV Proteins—Because milk composition is known to vary among individuals we determined the variation of identified proteins in milk-derived EV between individual donors. This revealed that a total of 367 proteins were shared among all seven donors, which is 19% of the total of 1963 unique proteins in the milk-derived EV proteome (Table III). Although variation exists whereby some proteins are uniquely identified in specific donors, a substantial number of proteins is shared between the different milk-derived EV samples, indicating that there is a common milk-derived EV proteome. Importantly, from the proteins shared among all donors, several are considered as EV-associated proteins and are used as markers for EV (as reviewed by Lötvall *et al.* (17)). These EV-associated proteins can be categorized according to their subcellular location (Table IV). For instance, transmembrane or lipid-bound extra-

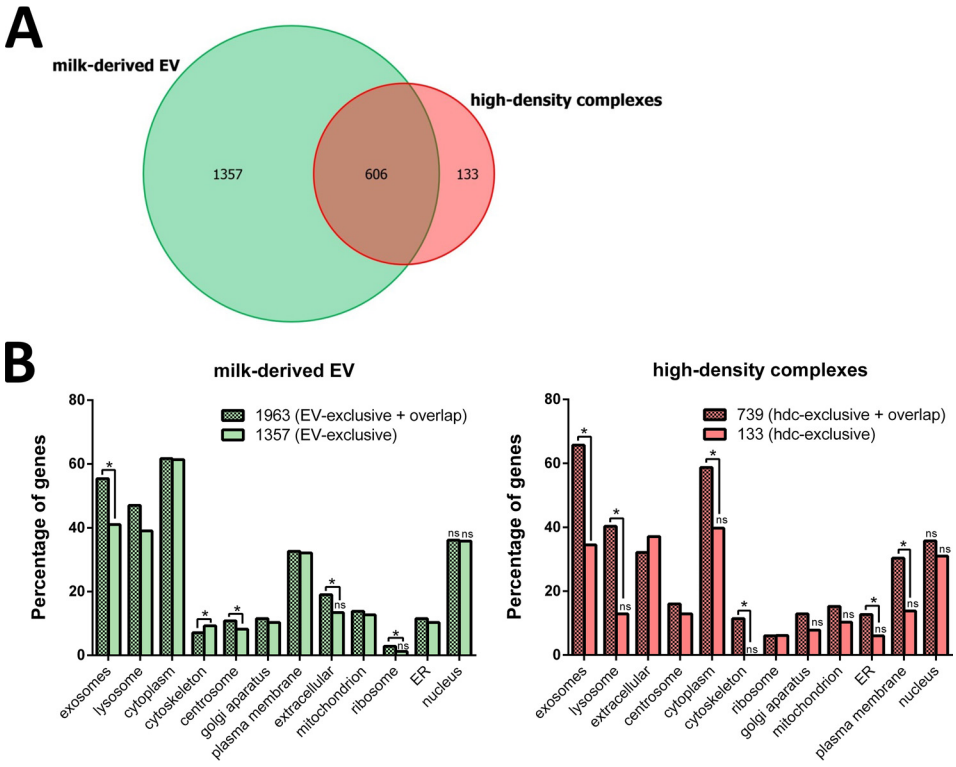

FIG. 2. **Comparison of the milk-derived EV and high-density complexes proteomes.** *A*, Venn diagram comparing uniquely identified and shared proteins between milk-derived EV and high-density complexes (pooled data from all seven milk donors). *B*, Functional annotations for cellular component of the identified proteins comparing all 1963 proteins identified in milk-derived EV *versus* 1357 proteins exclusively identified in EV (left figure). Additionally, all 739 proteins identified in the high-density complexes were compared with the 133 proteins exclusively identified in this sample. Enriched terms were ranked by *p* value (Hypergeometric test) for either data set. Those GO terms that were not significantly enriched are indicated with "ns." Within individual GO-terms, annotated proteins were compared for distribution via chi-square test. Those data sets that were significantly different are indicated with "*." For both the Hypergeometric test and the chi-square test, a *p* value <0.05 is regarded as significant.

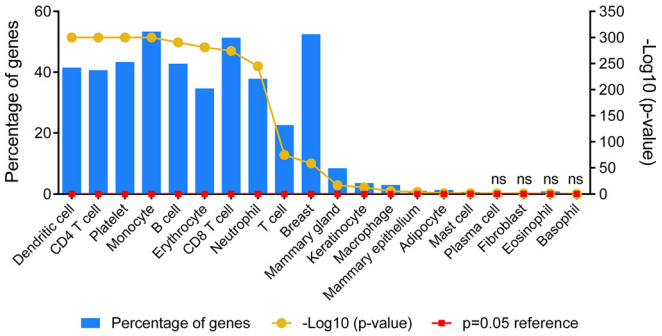

FIG. 3. **Site of expression of milk-derived EV proteins.** The 1963 proteins identified in all 7 milk-derived EV samples were analyzed for the site of expression. The percentage of genes linking to individual GO terms were ranked by *p* value and are shown, together with the *p* value from the Hypergeometric test (-Log10 depicted in yellow) and the reference $p = 0.05$ value (depicted in red). Those GO terms that were not significantly enriched are indicated with "ns."

TABLE III

The number of identified proteins shared among donors. The milk-derived EV proteomes from all seven individual donors were compared in an overlay. From these data the number of shared proteins between donors was calculated. Percentage of total relates to the total of 1963 unique proteins identified in all seven milk-derived EV samples

	Number of shared proteins	Percentage of total
Identified in all 7 donors	367	19
Identified in at least 6 donors	527	27
Identified in at least 5 donors	653	33
Identified in at least 4 donors	789	40
Identified in at least 3 donors	954	49
Identified in at least 2 donors	1245	63

cellular proteins (*e.g.* tetraspanins CD9, CD63, and CD81), and cytosolic proteins (*e.g.* annexins and Ras-related proteins) were identified in all milk-derived EV samples, whereas some intracellular proteins (*e.g.* histone) that are not expected to be enriched in EV were indeed lacking. In contrast to the milk-derived EV proteome, EV associated proteins are underrepresented or lacking in the proteome of the high-density complexes (Table IV). These data were confirmed by Western blotting (Fig. 4), which showed the presence of Flotillin-1 in milk-derived EV while being absent in high-density complexes. CD9 was more abundantly present in milk-derived EV than in high-density complexes.

TABLE IV

EV-associated proteins are preferentially identified in milk-derived EV samples. Different categories of proteins are present in EV, according to Lötvall et al. (17). Proteins that were identified in at least five out of seven donors in either sample (milk-derived EV, or high-density complexes) are shown, except for intracellular proteins not expected to be enriched in EV, these are given for all samples

Proteins	Full name	Milk-derived EV	High-density complexes
transmembrane or lipid-bound extracellular proteins (enriched in EV)			
CD9	CD9 antigen	present (7/7)	present (2/7)
CD63	CD63 antigen	present (7/7)	present (2/7)
CD81	CD81 antigen	present (7/7)	present (7/7)
ICAM1	Intercellular adhesion molecule 1	present (7/7)	present (1/7)
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	present (7/7)	-
FLOT1	Flotillin 1	present (7/7)	-
GNA11	Guanine nucleotide-binding protein subunit alpha-11	present (7/7)	present (1/7)
GNA13	Guanine nucleotide-binding protein subunit alpha-13	present (7/7)	-
GNAS	Guanine nucleotide-binding protein G(s) subunit alpha	present (7/7)	-
GNAQ	Guanine nucleotide-binding protein G(q) subunit alpha	present (7/7)	-
MFGE8	Lactadherin	present (7/7)	present (7/7)
cytosolic proteins (enriched in EV)			
ANXA2	Annexin A2	present (7/7)	present (6/7)
ANXA4	Annexin A4	present (7/7)	present (2/7)
ANXA5	Annexin A5	present (7/7)	-
ANXA6	Annexin A6	present (7/7)	present (1/7)
ANXA7	Annexin A7	present (7/7)	-
ANXA11	Annexin A11	present (7/7)	-
RAB5A	Ras-related protein Rab-5A	present (7/7)	present (1/7)
RAB7A	Ras-related protein Rab-7A	present (7/7)	present (3/7)
RAB11B	Ras-related protein Rab-11B	present (7/7)	-
RAB25	Ras-related protein Rab-25	present (7/7)	-
RAB35	Ras-related protein Rab-35	present (7/7)	present (2/7)
SDCBP	Syntenin-1	present (7/7)	present (2/7)
intracellular proteins (not expected to be enriched in EV)			
Hsp90B1	Endoplasmin	present (5/7)	present (3/7)
CANX	Calnexin	present (5/7)	present (1/7)
GM130	130 kDa cis-Golgi matrix protein	-	-
CYC1	Cytochrome C	-	-
HIST	Histone	-	-
AGO	Argonaute	-	-

Milk-derived EV Contain Proteins Not Listed in the Vesiclepedia Database—Apart from common EV proteins (Table IV), EV contain subset-specific proteins because of different cells of origin and biogenesis pathways. To identify any known EV proteins in milk-derived EV, we compared the 1963 proteins identified in the milk-derived EV with previously published EV studies included in the (human) Vesiclepedia database (25). Vesiclepedia contains 8450 unique proteins identified in EV from an extensive number of studies (currently 188 individual human studies) that included the characterization of EV from a wide variety of cellular sources (*e.g.* bodily fluids, primary cells, and cell lines) and vesicle types.

When comparing the overlap we found that 1765 proteins were already reported in Vesiclepedia (which is 90% of the 1963 proteins in the milk-derived EV proteome), however 198 proteins have been identified in this study that have not been listed in Vesiclepedia (Fig. 5*A*). With the results from this analysis, we can append the current "total" EV proteome to 8648 unique proteins.

Among the proteins that have not previously been reported in other EV were Oleoyl-ACP Hydrolase (OLAH) and Parathyroid Hormone-related Protein (PTHLH). In our proteomic analysis OLAH was detected with a coverage of 27,9% with five unique peptides, whereas PTHLH was detected with a coverage of 40,7% with nine unique peptides (supplemental File S1). Using Western blot analysis, we verified the presence of both proteins on all donors tested (Fig. 5*B*). Interestingly, various isoforms of PTHLH have been identified in human milk (26), which were detected with varying intensities between donors.

Milk-derived EV Contain Proteins Previously Not Identified in Milk Proteomic Studies—Breast milk is a complex body fluid that contains a variety of macromolecular structures that harbor proteins. The most frequently studied milk components abundant in proteins are MFG, caseins, and whey (6, 7, 27). Apart from whole milk and skim milk, the proteomes of milk fat globule membrane (MFGM), caseins and whey have been extensively characterized. Based on these data, we

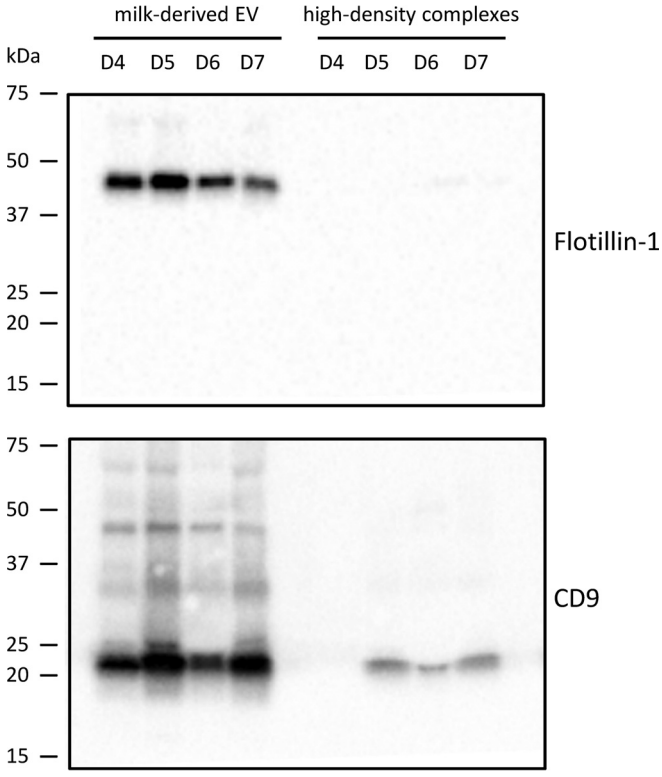

FIG. 4. **Western blot analysis on EV-associated markers.** The presence of flotillin-1 (~48 kDa) and CD9 (~23 kDa) was determined in isolated milk-derived EV or high-density complexes by Western blotting for donors 4, 5, 6, and 7.

constructed the human milk proteome by combining the data from eight previously published studies (22, 23, 28–33), which represent a total of 38 individual studies on whole milk, skim milk, caseins, whey, and MFGM (supplemental Table S1). This "total" milk proteome has a sum of 2698 unique proteins (supplemental File S2).

Next, we investigated whether the proteins identified in milk-derived EV have been identified in previous milk studies (because whole milk and skim milk will contain EV), or whether the differential centrifugation of milk followed by density gradient-based isolation of EV improved the detection of proteins that would otherwise be masked by highly abundant milk proteins. When comparing the milk-derived EV proteome from this study to the total milk proteome, 1330 proteins were shared (which is 49% of the total milk proteome of 2698 proteins) between milk-derived EV and the composed milk proteome (Fig. 6*A*). Interestingly, we identified 633 proteins that have never been described in human milk before. This indicates that isolation of EV as described in this study allows for a better separation of major milk components, which increases the dynamic range in which EV proteins can be detected. Our data reveal that human milk contains more unique proteins than previously known, with now 3331 unique proteins identified. Milk-derived EV contribute substantially to the total milk proteome, because 59% of the proteins (1963 of 3331 total proteins) have been identified in milk-derived EV. We confirmed the presence of two newly identified milk proteins using Western blotting. Milk-derived EV contained Myelin protein zero-like protein 1 (MPZL1) and EH domain-containing protein 3 (EHD3) (Fig. 6*B*).

Additionally, enrichment analysis was performed on the subcellular origin and the possible functions of the newly identified milk-derived EV proteins. We compared the 633 proteins exclusively identified in milk-derived EV to the 1368 proteins from the total milk proteome that are not shared with milk-derived EV (which we will refer to as non-EV milk proteins). We evaluated the cellular distribution of proteins by selecting for major cellular compartments. The 633 proteins exclusively identified in milk-derived EV proteins were significantly enriched for GO terms like "cytoskeleton," "plasma membrane," "cytoplasm," "lysosome," and "exosomes" (Fig. 6*C*). The 1368 non-EV milk proteins were significantly enriched for cellular component terms like "ER," "centrosome," "ribosome," and "extracellular." Subsequently, we compared the molecular function of proteins and found that complement, cytokine, and chemokine activity, and "translation regulator activity" was significantly enriched in the non-EV milk proteome. Interestingly, proteins exclusive to milk-derived EV are significantly enriched in other molecular functions *e.g.* cytoskeletal/structural activity, "transmembrane receptor protein tyrosine kinase activity," and "cell adhesion." Finally, the annotated biological processes of proteins was assessed. Although significantly enriched non-EV milk proteins were involved in "immune response" and "protein metabolism," the milk-derived EV proteins were enriched for biological processes related to, "signal transduction" and "cell growth and/or maintenance." Interestingly, the protein EHD3, which was verified with Western blotting (Fig. 6*B*), was annotated under signal transduction and "cytoskeletal protein binding," Together, the GO analysis indicates that proteins from milk-derived EV come from different cellular compartments and have other molecular functions and are involved in distinct biological processes compared with non-EV milk proteins.

DISCUSSION

To unveil the proteome of milk-derived EV, we performed a comprehensive proteomic analysis of purified EV isolated from breast milk from seven different donors. For EV isolation we used our recently established protocol optimized to purify EV from human milk (10). Proper sample collection and storage are critical for accurate analysis of EV that are naturally present in body fluids (34). For breast milk, removal of cells and MFG by differential centrifugation at low speed before long term storage is crucial (10). Next, isolation of EV from milk supernatant should involve density gradient based ultracentrifugation for further EV enrichment. An alternative approach often used for EV isolation is the direct pelleting of EV via ultracentrifugation (with at least 100,000 × *g*), however this approach yields less pure EV (35) and leads to an insol-

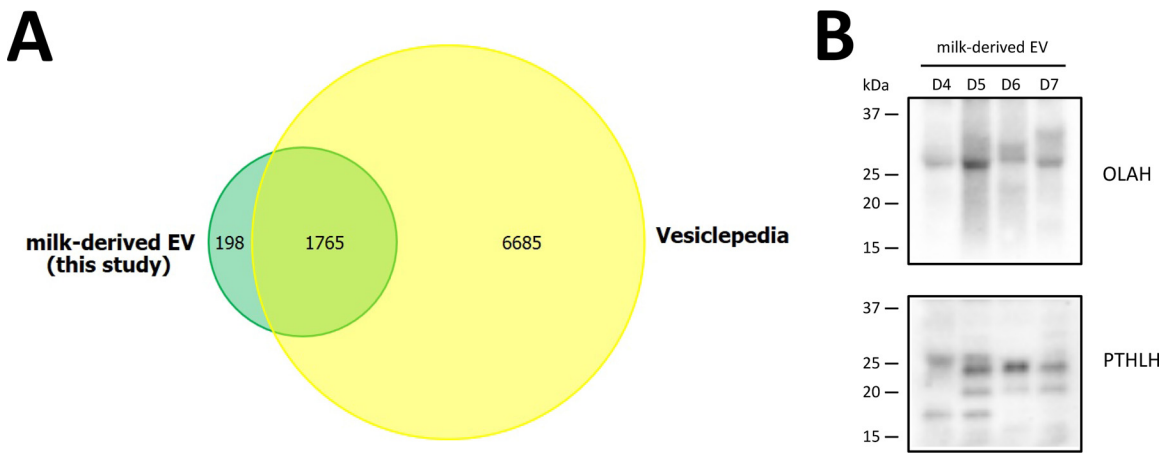

FIG. 5. **Comparison of the milk-derived EV proteome from this study to the EV database Vesiclepedia.** *A*, All protein entries from Vesiclepedia were downloaded and imported into FunRich for overlay analysis with the milk-derived EV proteome from this study, which is shown in the Venn diagram. *B*, Western blot analysis was performed for OLAH (~30 kDa) and PTHLH (~20 kDa and ~24 kDa) on milk-derived EV from donors 4, 5, 6, and 7.

uble pellet when working with breast milk (10). Therefore, top-down ultracentrifugation of milk supernatant is necessary and allows for an enhanced separation of EV from nonfloating complexes with high densities. Using this protocol, we isolated milk-derived EV and high density complexes from seven individual donors and performed proteomics analysis with high-resolution mass spectrometry. We identified a total of 1963 proteins in the milk-derived EV, whereas 739 proteins were identified in high-density complexes. In the milk-derived EV samples, common EV associated proteins were frequently present thereby confirming and supplementing earlier work in which CD9, CD63, MHC-class II, and Flotillin-1 were detected in milk-derived EV via Western blot (10).

Milk composition of an individual changes during lactation (7, 36–38). This is influenced by a variety of factors including diet (39) and age of the mother (40). We did observe that the milk-derived EV proteome from donor 2 was considerably larger than from the other donors (1615 proteins, whereas the average of the other donors was 814 proteins), whereas the high-density complex proteome was similar. We therefore performed additional GO analyses excluding donor 2 and found that this gave similar results and conclusions for all data presented in this report (data not shown). Using the current approach it remains to be determined whether during lactation the total number of EV changes, or that the composition of milk-derived EV alters. However, it is remarkable that the proteomes of the milk-EV from the individual donors, ranging in age from 32 to 39 and between 3 to 9 months after delivery, share 367 proteins (which is 19% of the total milk-derived EV proteome). This indicates that a part of the molecular composition of milk-derived EV is less influenced by these factors. A likely explanation for this finding is the presence of common EV proteins among these shared proteins. Furthermore, because we found that not only breast tissue contributes to the milk-derived EV pool, but that also immune cells are likely producers of milk-derived EV, changes in the EV proteome during lactation might be dependent on other factors than the one regulating soluble and nutritional milk components. It remains to be determined whether the total number of EV changes, or that the composition of milk-derived EV is altered during lactation.

Using the EV database Vesiclepedia (25), which included 8450 unique proteins identified in EV from a broad range of different origins, we were able to determine a large overlap of 1765 proteins between this milk-EV study and previously documented EV-proteins in Vesiclepedia, illustrating a common protein profile of EV regardless of the cell type that produces them, or bodily fluid from which EV are isolated. Additionally, this study reveals 198 proteins identified in milk-derived EV that have not been identified in other EV studies before. The discovery of these EV proteins could be because milk-derived EV are underrepresented in this database, because only few studies have characterized human milk-derived EV (12, 13, 41), contributing only 81 proteins to Vesiclepedia. Indeed, some of the proteins we identified in milk-derived EV that are not listed in Vesiclepedia, OLAH, and PTHLH, have been described in milk proteomics studies on whole milk or skim milk (22, 42–44). These proteins are thus specific to EV derived from milk (and have not been identified in other EV preparations). Moreover, OLAH could potentially be used to differentiate between milk-derived EV and MFG, as some studies performed on MFG have reported the presence of PTHLH, whereas OLAH has never been detected in this milk component (31–33, 37). Both OLAH and PTHLH have been detected in whey (36, 45), which contains EV but not MFG, further supporting the idea that milk-derived EV express both OLAH and PTHLH. Most likely, OLAH and PTHLH will be derived from the EV subset produced by cells in the mammary gland, because these proteins were exclusively mapped to "breast" and "mammary gland" respectively in the GO anal-

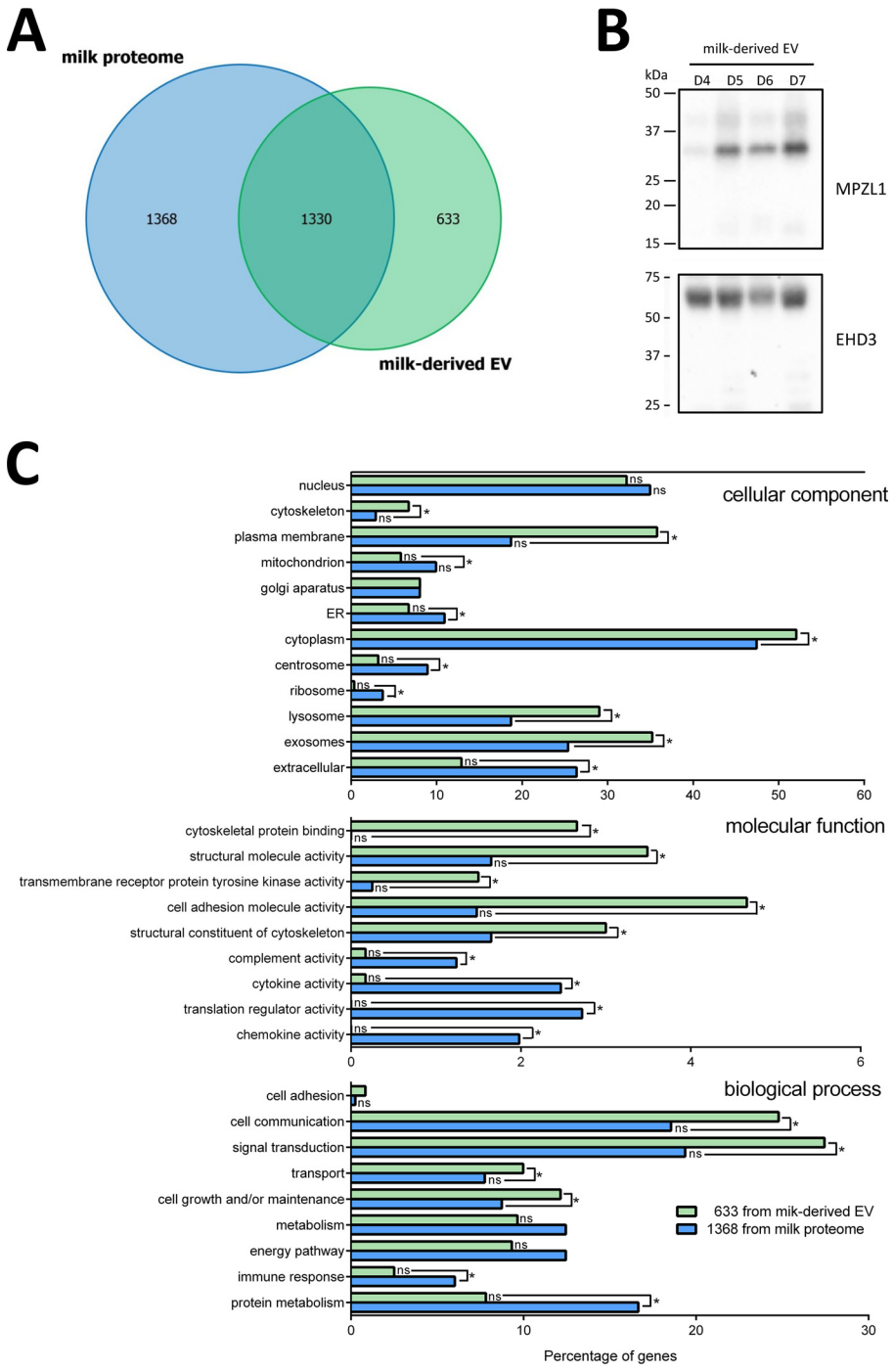

FIG. 6. **Comparison of the milk-derived EV proteome to the milk proteome.** *A*, Comparison of the proteins identified in milk-derived EV from all 7 donors in the current study with the milk proteome constructed from previously published milk studies. Thirty-eight published (or reviewed) proteomes described in Beck (22), D'Alessandro (23), Dallas (28), Guerrero (29), Hettinga (30), Spertino (31), Yang (32) and Lu (33) were combined such that each protein is represented by its common gene name. The milk proteome was compared with the milk-derived EV proteome from this study and is depicted in the Venn diagram. *B*, MPZL1 (~29 kDa) and EHD3 (~61 kDa) were detected on milk-derived EV from donors 4, 5, 6, and 7 using Western blot analysis. *C*, Functional annotations of proteins uniquely identified in milk-derived EV compared with non-EV milk proteins. The 633 proteins exclusively identified in milk-derived EV (shown as milk-derived EV in green) were compared with the 1368 proteins from the milk proteome that were not shared with milk-derived EV (shown as milk proteome in blue). Enriched terms for cellular compartment, molecular function and biological process term were ranked by *p* value (Hypergeometric test) for either data set. Those GO terms that were not significantly enriched are indicated with "ns." Within individual GO-terms, annotated proteins were compared for distribution via chi-square test. Those data sets that were significantly differently distributed are indicated with "*." For both the Hypergeometric test and the chi-square test, a p value <0.05 is regarded as significant.

ysis for site of expression in this study. However, besides EV produced by cells in the mammary gland we found by GO analysis that also immune cells might be the origin of milk-derived EV. Taken together the cellular origin of milk-derived EV is diverse and it remains to be determined what the contribution of individual cell types is to the number and composition of milk-derived EV.

Although the analysis of breast milk composition has received considerable attention, milk-derived EV have not yet been recognized as a major component of milk. In order to compare and assess the relative contribution of the milk-derived EV proteome to proteins previously identified in other milk components, we combined the data from previous milk studies (including analysis of whole milk, skim milk, casein, whey and MFG) to construct the proteome of total human milk. Overlap analysis revealed that 1330 proteins that were identified in our study have also been identified in other milk proteome studies and reflect 49% of the total milk proteome. Because the total milk proteome contains proteins identified in whole milk and skim milk, which both contain milk-derived EV, indeed a considerable overlap was expected. Why in previous milk proteomics studies the milk-derived EV proteome was not completely unrevealed could be caused by the relative low number of EV in the whole milk analyzed. This is in contrast to our study, where EV were purified and concentrated. Additionally, milk-derived EV contain proteins also present in other milk components. For instance, CD9 and MUC-1 have also been identified in MFG (37). Because the isolation procedure of MFG is considerably different from milk-derived EV the most likely explanation for this finding is that the EV-proteins are not derived from contaminating EV in the MFG fraction but that these proteins are indeed present in both macromolecular components. Finally, in the 49% overlap between previous milk studies and this EV study, also highly abundant proteins in milk, like caseins or lactoferrin are present. These proteins are often identified in proteomic studies of milk regardless of the isolation procedure aimed to purify a specific milk component.

Remarkably, 633 milk-derived EV proteins from this study have not been reported in any of the 38 previous milk proteomics studies. The fractionation of the milk with our established protocol to purify EV, using differential centrifugation followed by density gradient ultracentrifugation, allows for an increased dynamic range in detection, because abundantly present milk proteins that would otherwise mask the detection of low abundant proteins are separated from the milk-derived EV. Importantly, this study demonstrates that milk contains much more different proteins than previously known and that a substantial number of these proteins is EV-associated. Remarkably, whereas the proteins of the total milk proteome that are not shared with milk-derived EV are primarily linked to nutritional pathways (reflecting the nutritional characteristics of milk) and immune function based on complement activity and cytokine/chemokine function, the unique milk-derived EV proteins are linked to other bioactive biological pathways like cell signaling and cell growth and/or maintenance.

In conclusion, by using an optimized EV isolation procedure together with in-depth proteomic analysis we unraveled the milk-derived EV proteome in detail and identified novel bioactive proteins associated with milk-derived EV. This indicates that besides commonly described milk components, milk-derived EV need to be recognized as yet another important bioactive macromolecular component in breast milk, which might be involved in transmitting signals from the mother to the newborn.

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